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Toward Diagnosing *Leishmania infantum* Infection in Asymptomatic Dogs in an Area Where Leishmaniasis Is Endemic▽

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The most frequently used diagnostic methods were compared in a longitudinal survey with *Leishmania infantum*-infected asymptomatic dogs from an area of Italy where leishmaniasis is endemic. In February and March 2005, 845 asymptomatic dogs were tested by an immunofluorescence antibody test (IFAT), a dipstick assay (DS), and an enzyme-linked immunosorbent assay (ELISA) for *L. infantum* and by IFAT for *Ehrlichia canis*. Dogs seronegative for *L. infantum* were further parasitologically evaluated by microscopic examination of lymph node tissues and PCR of skin samples. A total of 204 animals both serologically and parasitologically negative for *L. infantum* at the first sampling were enrolled in the trial and were further examined for canine leishmaniasis (CanL) and canine monocytic ehrlichiosis in November 2005 (i.e., the end of the first sandfly season) and March 2006 and 2007 (1- and 2-year follow-ups, respectively). At the initial screening, the overall rates of *L. infantum* seroprevalence were 9.5% by IFAT, 17.1% by ELISA, and 9.8% by DS and the overall rate of *E. canis* seroprevalence was 15%. The rates of concordance between the results of IFAT and DS were almost equal, whereas the rate of concordance between the results of IFAT and DS and those of the ELISA was lower. The results of the annual incidence of *Leishmania* infection were variable, depending on the test employed, with the highest values registered for PCR (i.e., 5.7% and 11.4% at the 1- and 2-year follow-ups, respectively), followed by ELISA, IFAT, and DS. Over the 2 years of observation, 55 animals (i.e., 26.9%) became positive for *L. infantum* by one or more diagnostic tests at different follow-up times, with 12.7% showing clinical signs related to CanL, while the remaining 87.3% were asymptomatic. A diagnostic scheme for assessment of the *L. infantum* infection status in asymptomatic dogs is suggested.

Canine leishmaniasis (CanL) due to *Leishmania infantum* is transmitted by different species of *Phlebotomus* sandflies and is considered one of the most important canine protozoal diseases of zoonotic concern (2). *L. infantum* is widely distributed in many Mediterranean countries; and in Italy, stable endemic foci consisting of dogs from the central and southern areas of the country have been reported (4, 5, 30, 32), with high percentages (up to 53.1%) of animals being serologically positive (5). More recently, *L. infantum* infections have spread throughout the northern regions of Italy (19). In central Europe, CanL is a well-known and emerging travel-associated disease, and the occasional focal autochthonous transmission of *Leishmania* has been suspected (16). In dogs, infections may cause severe clinical forms or dogs may remain asymptomatic for a long time (5, 7, 17). Many clinical features of CanL (e.g., lethargy, weight loss, anorexia, epistaxis, lymphadenomegaly, and splenomegaly) may be similar to those of other diseases, including canine monocytic ehrlichiosis (CME) (13). Indeed, along with CanL, CME caused by *Ehrlichia canis*, which is transmitted by the brown dog tick (*Rhipicephalus sanguineus*), is an important canine vector-borne disease in several countries of the Mediterranean basin (40). In areas of endemicity, CanL may represent a veterinary and public health issue mainly due to the high percentage of asymptomatic animals (up to 85%) in areas of endemicity (7); and these asymptomatic animals, like symptomatic ones, may serve as reservoirs for the vector-borne transmission of *Leishmania* spp. to receptive animals and humans (22, 24). Thus, the reliable identification of *Leishmania*-infected asymptomatic reservoir animals is crucial for any successful control strategy.

The definitive diagnosis of *Leishmania* infection in asymptomatic animals is troublesome, since both serological and parasitological methods have inherent limitations (23). Indeed, serology may not be a good indicator of infection when it is used in cross-sectional studies due to the various times that span between infection and seroconversion (i.e., from 3 months to 7 years [1]). Additionally, asymptomatic infected animals may remain seronegative as a consequence of their individual immune response (3). Among the direct parasitological tests, microscopic examination is a rapid and simple method; but it has a low sensitivity, particularly with asymptomatic dogs, and thus, it is not recommended for use for mass screenings in areas of endemicity. Although in vitro culture techniques are reliable and sensitive, they are prone to microbiological contamination (12), especially if skin samples or samples collected under field conditions are used. Molecular tools that detect *Leishmania* DNA in putative dog reservoirs have been developed (33), and they have been shown to be

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more sensitive than serology and culture techniques (10). Thus, although PCRs can be useful for the detection of asymptomatic infected animals (10, 27), definitions of the methodologies, amplification protocols, and gene targets to be used and the tissue type to be tested are a matter of debate among scientists (6, 36).

As a consequence, data currently available in the literature about diagnosis of CanL in asymptomatic animals are controversial, and a diagnostic "gold standard" is far from being clearly stated (23). Again, no longitudinal studies are available to investigate the serological and parasitological features that appear over the course of the first *L. infantum* infection in asymptomatic animals from an area of endemicity for canine vector-borne diseases. Thus, it was the aim of the present study to compare the most frequently used diagnostic methods in a longitudinal survey of *L. infantum*-infected asymptomatic dogs from areas of endemicity.

MATERIALS AND METHODS

Study design. From January 2005 to March 2006, samples were collected from dogs in the context of a field trial described previously (29), with further sampling taking place in March 2007 (see below). All animals were housed in two kennels of the Apulian region in southern Italy (latitude, between 42°N and 39°N; longitude, between 15°E and 18°E) where CanL is endemic and had been reported over the previous 2 years (30). The animals were kept under their usual housing conditions (i.e., housing, food, temperature regulation, and ventilation) (29). The animals were handled and sampled with the owners' consent and with the approval of the Ethical Committee of the Faculty of Veterinary Medicine of the University of Bari (Bari, Italy). The sex, age, weight, and coat length were recorded for each dog.

Briefly, in January and February 2005 (i.e., in the absence of sandflies in the study area [18]), about 2,000 dogs were clinically evaluated. A total of 845 dogs of both sexes and different ages that did not show any signs of dermatitis or lymphadenomegaly were further tested by an immunofluorescence antibody test (IFAT), an immunochromatographic dipstick assay (DS), and an enzyme-linked immunosorbent assay (ELISA) for the presence of specific anti-*Leishmania* antibodies and by IFAT for the presence of specific anti-*E. canis* antibodies. Dogs without detectable anti-*Leishmania* antibodies were further examined for the presence of amastigote stages of *Leishmania* parasites in stained lymph node smears and for *Leishmania* DNA in dermal tissue samples by PCR (see below).

In March 2005, 204 animals (i.e., 102 from each kennel) which were both serologically and parasitologically negative for *L. infantum* were enrolled in a longitudinal follow-up trial, irrespective of the presence of anti-*E. canis* antibodies. All animals remained untreated with any ectoparasiticide through the study. They were examined serologically and parasitologically for CanL and CME in November 2005 (i.e., the end of the first sandfly season), March 2006 (1-year follow-up), and March 2007 (2-years follow-up). Specifically, serological tests for the presence of anti-*Leishmania* antibodies (IFAT, ELISA, DS) and anti-*E. canis* antibodies (IFAT) as well as PCR for the detection of *Leishmania* DNA in dermal tissue samples were performed at all follow-up times. Additionally, lymph node tissue smears were examined microscopically in March 2006 and 2007 for the presence of amastigote *Leishmania* stages. Clinical examinations were performed monthly, and clinical signs (i.e., lethargy, weight loss, anorexia, alopecia, dermatitis, conjunctivitis, epistaxis, onychogryposis, lymphadenomegaly, etc.) were recorded.

Diagnostic procedures. (i) Serological tests. Three different serological tests were used to reveal specific anti-*Leishmania* IgG antibodies.

***(a)* IFAT.** IFAT was performed with promastigotes of *L. infantum* zymodeme MON1 as the antigen. The cells were exposed to sera diluted (1:80) in phosphate-buffered saline in a moist chamber and then to fluoresceinated rabbit anti-dog immunoglobulin G (IgG) serum diluted 1:40 (rabbit anti-dog IgG; lot 125K4752; Sigma-Aldrich Chemie, Germany); each exposure was at 37°C for 30 min. The samples were scored positive when they produced a clear cytoplasmic or membrane fluorescence with promastigotes by use of a cutoff dilution of 1:80. Positive sera were titrated until they gave negative results.

***(b)* ELISA.** The ELISA was performed by using water-soluble proteins of promastigote forms of *L. infantum* (zymodeme MON1) as antigens and goat anti-dog IgG antibodies (gamma chain specific) conjugated to alkaline phosphatase (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) as detection antibodies. Samples were considered positive if the absorbance at 405 nm (A_{405}) was above the arithmetic mean plus 3 standard deviations of the A_{405} values for 48 *Leishmania* control dogs originating from an area in southern Switzerland free of *L. infantum*, *E. canis*, and *Babesia canis* (21). The results were expressed as a system of arbitrary antibody units (AU), where 0 AU corresponds to the threshold value and 100 AU corresponds to the result for the positive standard serum sample.

***(c)* DS.** A commercially available DS (*Leishmania* RapydTest; product code 1603, lots CF1079 and GK1062; DiaSys Europe Ltd., Wokingam, United Kingdom), based on the rK39 antigen and validated for use with samples from dogs (28), was performed according to the manufacturer's instructions.

E. canis-specific antibody were detected by IFAT by using slides containing fixed *E. canis* parasites in DH82 cells (Canine Ehrlichiosis FA substrate slide; lot P060228-002032908; VMRD, Pullman, WA). The parasitized cells were exposed to sera diluted (1:50) in phosphate-buffered saline (pH 7.2) in a moist chamber, and after the cells were washed, they were exposed to fluoresceinated rabbit anti-dog IgG (rabbit anti-dog IgG; lot 125K4752; Sigma-Aldrich Chemie) diluted 1:60; both incubations were done at 37°C for 30 min. Samples were scored positive when cytoplasmic inclusion bodies produced fluorescence at a dilution of 1:50 or higher. All positive serum samples were further titrated until the reaction became negative.

(ii) Parasitological diagnosis of CanL. Tissue from the popliteal lymph nodes was sampled by using a nonaspiration technique (20). Lymph node tissue smears were stained with Diff Quick (lot 100510; Medical Team Srl, Italy) and were microscopically examined for the presence of amastigote stages (1.5 to 2.0 by 2.5 to 5 μm) of *Leishmania* parasites.

One skin sample weighing about 30 mg per animal and per collection time was taken from the right shoulder region by using a disposable ophthalmology scalpel after the hair over an area of about 0.5 by 0.5 cm was clipped. The samples were stored at −20°C in Eppendorf tubes containing 1 ml of phosphate-buffered saline.

After disruption in liquid nitrogen and pestling (i.e., two freeze-thaw cycles), genomic DNA was extracted from the approximately 30-mg skin samples by using a commercial kit (genomic DNA purification kit; Gentra Systems). An *L. infantum* kinetoplastid minicircle DNA fragment was amplified with the MC1-MC2 primer pair (6). Genomic DNA solution (4 μl) was added to the PCR mixture (46 μl), which contained 2.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 250 μM of each deoxynucleoside triphosphate, 50 pmol of each primer, and 1.25 U of Ampli *Taq* Gold (Applied Biosystems, Milan, Italy). Optimal conditions for PCR amplification were standardized as follows: initial denaturation at 94°C for 12 min; 30 cycles consisting of denaturation at 94°C for 30 s, annealing at 60°C for 20 s, and extension at 72°C for 30 s; and a final extension at 72°C for 5 min. A positive control containing genomic *L. infantum* DNA and a negative control without DNA were included in all the assays. Amplification products (~447 bp) were visualized on 2% (wt/vol) agarose gels (Ambion, Milan, Italy) upon staining with ethidium bromide.

Statistical analysis. (i) Sample size. The minimum sample size ($n = 128$, i.e., 64 dogs from each kennel) for estimation of incidence was calculated by using WinEpiscope (version 2.0) software (39) and by making the following assumptions: the dog population for each kennel ($n = 800$), an expected incidence of 11% (29), maximum accepted error of 5%, and a confidence level of 95%. Since a certain number of dogs must be expected to be lost to follow-up during a long study, especially in the kennel situation, more than 100 dogs instead of 64 dogs were enrolled in each group.

(ii) Incidence calculation and test performance. The crude incidence at each sampling date and for each test was calculated as the proportion of positive dogs in comparison to the size of the susceptible population. For each calculation, dogs previously positive by the same test were excluded and no longer contributed to the incidence calculation, although they were tested each time. The yearly incidence and the apparent recovery rates (the percentage of dogs that were positive and the percentage of dogs that were negative 1 year later by use of the same test) were calculated in March 2006 and 2007 and in March 2007, respectively, regardless of the results obtained in November 2005.

The rates of concordance among the serological tests performed in the initial screening (March 2005) with asymptomatic dogs was evaluated by the use of kappa statistics (39). Kappa values were ranked as low ($0.2 < \kappa < 0.4$), moderate ($0.4 < \kappa < 0.6$), good ($0.6 < \kappa < 0.8$), or excellent ($\kappa > 0.8$), as suggested by Everitt (8). At the follow-up examinations (November 2005, March 2006, and March 2007), the relative sensitivity (SE_{rel}) and the relative specificity (SP_{rel}) of the serological tests were assessed on the basis of the PCR results obtained with samples obtained at the same sampling time. Sensitivity and specificity were calculated as described by Greiner and Gardner (11).

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TABLE 1. Detection of anti-*Leishmania infantum* antibodies by different tests at the study starting point as well as anti-*Ehrlichia canis* antibodies in dogs with or without detectable anti-*L. infantum* antibodies

Test	No. of dogs positive/total no. of dogs tested (%)		
	Anti-*Leishmania infantum* antibodies	Anti-*Ehrlichia canis* antibodies[a]	
		L-pos dogs	L-neg dogs
IFAT	79/831 (9.5)	14/79 (17.7)	106/752 (14.1)
ELISA	143/837 (17.1)	38/143 (26.6)[b]	86/694 (12.4)[b]
DS	83/845 (9.8)	14/83 (16.9)	112/762 (14.7)

[a] L-pos dogs, dogs with detectable anti-*L. infantum* antibodies; L-neg dogs, dogs without detectable anti-*L. infantum* antibodies.
[b] $P < 0.001$.

TABLE 2. Measure of agreement among the results of IFAT, ELISA, and DS *Leishmania* tests calculated for 845 dogs at the study starting point[a]

Test and result	No. of dogs with the following DS result:		No. of dogs with the following ELISA result:	
	Negative	Positive	Negative	Positive
IFAT negative	750	2	692	54
IFAT positive	3	76	0	79
ELISA negative	694	0		
ELISA positive	63	80		

[a] For IFAT versus DS, the concordance of the results was excellent ($\kappa = 0.965$; $P < 0.001$); for IFAT versus ELISA, the concordance of the results was good ($\kappa = 0.710$; $P < 0.001$); and for ELISA versus DS, the concordance of the results was good ($\kappa = 0.678$; $P < 0.001$).

Determination of whether there was an association between the PCR results and the numbers of AU obtained by ELISA was screened for by one-way analysis of variance after conversion of the AU in log units (data + 1) to normalize the AU distribution; determination of whether there was an association between the PCR results and the titer obtained by IFAT was screened for by the chi-square test after categorization of the titer.

Statistical calculations were performed with the statistical package SPSS, version 13.0 for Windows, and WinEpiscope, version 2.0 (available at http://www.clive.ed.ac.uk/winepiscope/).

RESULTS

Initial screening. The results of the serological tests carried out at the initial screening of 845 dogs are summarized in Table 1. The overall seroprevalence rate (positive reaction by one or more tests) for anti-*L. infantum* antibodies was 17.3%, and that for anti-*E. canis* antibodies was 15% (data not shown). The prevalence of anti-*E. canis* antibodies was similar regardless of whether the serological status for *Leishmania* antibodies was assessed by IFAT or DS (range, 14.1% to 17.7%), but it was significantly higher (χ^2 test, $P < 0.01$) for ELISA-positive dogs (26.6%) than for ELISA-negative dogs (12.4%) (Table 1).

Parasites were detected microscopically in stained lymph node tissue smears of 3 (i.e., 0.7%) of 453 dogs. These three dogs were positive by serological tests. The rate of concordance between the results of IFAT and those of DS was almost perfect (kappa = 0.965; rating, excellent), with the results for only five cases being discordant, whereas the rate of concordance between the results of IFAT and the results of ELISA was lower (54 discordant cases; kappa = 0.71; rating, good). The rate of agreement between the results of ELISA and those of DS was also good (63 discordant cases; kappa = 0.678) (Table 2).

Follow-up. The 204 dogs negative for *Leishmania* by all diagnostic tests and thus enrolled into the longitudinal study represented a population homogeneous for sex, age, weight, and hair length (χ^2 test, $P > 0.05$), and among these dogs, 28 dogs (i.e., 13.7%) were positive for *E. canis* antibodies. Complete 2-year data sets (all tests, all sampling dates) were available for 173 dogs.

The incidences of CanL and CME calculated for each sampling date and diagnostic technique are summarized in Table 3. The test-specific annual incidence rates of *Leishmania* infections calculated for the periods of March 2005 to March 2006 and March 2006 to March 2007 were 5.7% and 11.4%, respectively, for PCR; 5% and 5.9%, respectively, for ELISA; 2.6% and 6.4%, respectively, for IFAT; and 2.1% and 0%, respectively, for DS (Table 3).

The SE_{rel} and SP_{rel} values of the serological tests determined on the basis of the results of PCR with skin tissue specimens showed considerable variation by diagnostic technique and sampling date (Table 4). In general, the test SE_{rel} values were low (less than 50%), but the test SP_{rel} values were high and ranged from 92 to 100%. The SP_{rel} values did not change with time, while the SE_{rel} values showed significant variations among tests and sampling times. In particular, the maximum SE_{rel} for all tests was recorded in March 2006 and the lowest one was recorded after 1 year, in March 2007. The SE_{rel} of the ELISA was significantly higher than the SE_{rel} values of IFAT and DS for the November 2005 and March 2006 sampling dates. Of five dogs classified as having doubtful results by DS (data not shown), three were negative by all the other *Leishmania* and *Ehrlichia* diagnostic tests at each follow-up, while the remaining two were clearly positive for anti-*E. canis* antibodies (antibody titer range, 1:200 to 1:1,600).

Throughout the 2 years of observation, 55 animals (i.e., 26.9%) tested positive for *L. infantum* by at least one of the diagnostic tests (Table 5). In particular, 28 animals were positive by only one of the parasitological tests (i.e., PCR or microscopy; group A), while 18 were serologically positive by one or more tests (group B). Only nine dogs were both parasitologically and serologically positive (group C). Of the 18 serologically positive animals in group B, 9 were positive by the ELISA only.

After the first sandfly season in November 2005, 25 animals were positive by one or more tests. Among those animals, 11 dogs were positive by the PCR only, 11 were positive by one of the serological tests only (for ELISA, $n = 9$; for IFAT, $n = 2$), and 3 were both parasitologically and serologically positive. The remaining 30 animals became positive by at least one of the parasitological and/or serological tests at subsequent sampling times (i.e., on March 2006 or March 2007) (Table 5).

At the final follow-up date (March 2007), only 7 (4%) of the remaining 173 animals showed symptoms of leishmaniasis (i.e., dermatitis, lymphadenopathy, conjunctivitis, or skin ulcers) (Table 5). Two among them belonged to group A and group B, respectively, while the remaining five animals belonged to group C. The 32 dogs ELISA positive throughout the observation period showed AU levels that ranged from 0.08 to 96.79.

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TABLE 3. Prevalence at starting point and incidence rates of *Leishmania infantum* and *Ehrlichia canis* infection calculated by each diagnostic technique

Parameter and date	% Incidence[a] (no. of dogs positive/total no. of dogs)				
	Leishmania				IFAT for *Ehrlichia*
	ELISA	IFAT	DS[b]	PCR	
Prevalence at starting point (March 2005)	0 (0/204)	0 (0/204)	0 (0/204)	0 (0/204)	13.7 (28/204)
Intermediate incidence determination					
March 2005–November 2005	5.1 (9/178)	2.0 (4/196)	0 (0/196)[c]	7.2 (14/195)	11.9 (20/168)
November 2005–March 2006	2.0 (3/149)	1.1 (2/187)	2.2 (4/179)[d]	0.6 (1/178)	3.4 (5/146)
Yearly incidence rate					
March 2005–2006	5.0 (8/159)	2.6 (5/192)	2.1 (4/192)	5.7 (11/193)	13.8 (25/176)
March 2006–2007	5.9 (9/153)	6.4 (11/173)	0 (0/168)	11.4 (19/166)	9.3 (13/139)
Yearly recovery rate[e] (March 2006–2007)	33.3 (2/6)	25 (1/3)	All	60 (6/10)	25.6 (10/39)

[a] Data for dogs previously positive by the same test were excluded from the calculation.
[b] Doubtful results were considered negative.
[c] The results for 13 dogs were doubtful.
[d] The results for four dogs were doubtful.
[e] The rate was calculated at the number of dogs negative in 2007/number of dogs positive in 2006.

Only five of these dogs were PCR positive and had significantly higher ELISA titers ($P < 0.05$). In particular, four PCR-positive dogs showed greater than 7.5 AU by ELISA, while only one dog had 0.73 AU. Of 22 IFAT-positive dogs, 7 were also PCR positive, and the results showed no statistical association with the titers. In particular, four PCR-positive dogs were IFAT positive; one dog had a titer of 1:80, one dog had a titer of 1:160, and two dogs had titers of 1:320.

DISCUSSION

The results of the present work clearly imply that the diagnosis of early CanL and the identification of asymptomatic carrier animals in an area of endemicity might be complex tasks. Indeed, at the initial screening, while the rates of seropositivity for *L. infantum* were similar by IFAT and DS (i.e., about 9.5%), the rate was higher when it was determined by ELISA (i.e., ~17%). Both, IFAT and DS were less sensitive than ELISA for the detection of specific antibodies in asymptomatic dogs without other infections and kept outside of an area of endemicity (21). In the present study, which focused on early infections in animals living in an area of endemicity, ELISA proved to be the most sensitive serological test on two occasions (November 2005 and March 2006), whereas no significant differences between the serological tests were noted in March 2007.

The significantly higher percentage of positive ELISA results for dogs also positive for *E. canis* antibodies (Table 1) indicates some kind of interaction between the two tests. One possible explanation is a cross-reaction of the ELISA that results in false-positive results for *Leishmania*, or it is also possible that *Leishmania*-specific antibodies detectable only by the ELISA might be responsible for false-positive reactions in the *Ehrlichia* IFAT.

Nonetheless, *E. canis* infection might induce immunosuppression and therefore might potentially increase the susceptibility of the dogs to *L. infantum* infection (14). It is broadly accepted that the threshold level of any serological test should be adapted to meet the local conditions. This is reflected by the finding that in our study 44.7% of the serum samples positive only by ELISA at the initial sampling had levels only marginally above the cutoff level. The concordance between the results of IFAT and DS ($\kappa = 0.965$) was high, with only a few cases of discordant results (Table 2). However, IFAT is also a quantitative test which could provide useful information about the immune reactivity of *L. infantum*-infected individuals. The rK39 DS represents an alternative to the currently available diagnostic tests, especially when it is used in mass screening surveys in which antibody titers are not required, and it is ideal for use under field conditions. Again, DS was validated against IFAT by using sera from dogs that were positive or negative by lymph node tissue smear parasitological examination and that thus had a visceral generalized form of infection if they were smear positive (28). Accordingly, by comparing the results of ELISA, IFAT, and the rK39 DS, DS was mainly shown to be helpful for confirming clinically suspected cases, while it was not very sensitive for the detection of asymptomatic infections (21). Consequently, for asymptomatic animals from areas of endemicity, a negative serological DS result may result from a subclinical infection and a positive result may occur for dogs coinfected with *E. canis* (28). Thus, the results of all serological tests should be evaluated carefully according to the epidemiological context of the area and the aim of the investigation. In addition, in asymptomatic animals a low level of humoral reactivity may lead to low antibody concentrations and, ulti-

TABLE 4. SE_{rel} and SP_{rel} of IFAT, ELISA, and DS serological tests for *Leishmania* compared to the results of PCR with skin tissue samples from asymptomatic dogs[a]

Test	November 2005		March 2006		March 2007	
	SE_{rel} (%)	SP_{rel} (%)	SE_{rel} (%)	SP_{rel} (%)	SE_{rel} (%)	SP_{rel} (%)
ELISA	$33^{*,\S,\P}$	95	$50^{\dagger,\ddagger,\S,\P}$	95.5	$9^{\P,\|\|}$	93
IFI	$14^{*,**}$	99	$27^{\dagger,**,\dagger\dagger}$	99	$13^{\dagger\dagger}$	92
DS			$18^{\|\|,\ddagger\ddagger}$	99	$4.5^{\ddagger\ddagger}$	100

[a] Equal letters correspond to a significant difference ($P < 0.01$).

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TABLE 5. Diagnostic follow-up of the 55 dogs positive for *Leishmania infantum* infection by one or more tests

Group and no. of dogs	Result by the indicated assay at the following times:													
	November 2005				March 2006					March 2007				
	IFAT	DS	ELISA	PCR	IFAT	DS	ELISA	Microscopy	PCR	IFAT	DS	ELISA	Microscopy	PCR
Group A (n = 28), parasitologically positive animals														
15														+
4[a]				+					+					
3				+					+					+
3				+										
2													+	+
1								+	+					
Group B (n = 18), serologically positive animals														
5												+		
2			+											
2			+				+							
3			+				+			+		+		
1										+				
1			+							+		+		
1							+							
1[a]			+							+				
1										+		+		
1	+				+	+				+		+		
Group C (n = 9), parasitologically and serologically positive animals														
2[a]										+			+	
1[a]				+	+			+	+			+		
1	+			+										
1												+		+
1	+			+	+	+			+	d[b]	d	d	d	d
1[a]			+	+	+	+	+		+	+	+	+	+	+
1[a]	+				+	+				+		+		+
1[a]								+		+			+	

[a] Single animals which showed symptoms of leishmaniasis (i.e., dermatitis, lymphadenopathy, conjunctivitis, or skin ulcers) and/or hypergammaglobulinemia in each line.
[b] d, dead animals.

mately, to borderline titers which may result in false-negative or -positive results due to cross-reactivity (15).

Compared to spleen tissue and bone marrow, popliteal lymph node tissue has been shown to be the most suitable for use for the direct parasitological detection of *L. infantum*-infected symptomatic animals (25). Conversely, in asymptomatic animals, at the initial screening, cytology showed a very low rate of positivity (i.e., 0.7%) and a low sensitivity compared to the results of serological techniques, as has already been demonstrated with animals from areas of endemicity (34). Thus, cytological analysis of lymph node tissue is not useful for the diagnosis of *Leishmania* infection in asymptomatic animals, since protozoa may remain at the dermal site or may be present at a low parasitic load (15) as a consequence of an effective immune response (31). Similarly, of the 55 animals newly infected at one or more follow-up evaluations, only 9 (i.e., 16.3%) were positive by the cytological examination, which indicates that cytology should be not the first parasitological test of choice for the diagnosis of asymptomatic *L. infantum* infections in animals. Again, the difficulty with sample collection from lymph nodes that are not enlarged impairs the use of cytology for asymptomatic animals. Interestingly, five of the nine animals positive by the cytological examination showed clinical symptoms, while three others became positive only at the final follow-up (Table 5), and thus, it was not possible to evaluate the dogs for the appearance of clinical signs. Only one dog positive by cytology and PCR in March 2006 became negative by all tests at the last follow-up.

In recent decades, different PCR protocols have been shown to be sensitive and specific for the diagnosis of CanL by the use of a variety of animal tissues (i.e., bone marrow, lymph nodes, skin, and blood) (9). A number of studies investigated different gene markers and protocols for the diagnosis of CanL, but in many cases the results were discordant. PCR with skin tissue samples (from any collection time) was positive for 34 (i.e., 61.8%) of 55 infected animals. Although PCR with skin tissue samples cannot be considered a gold standard for the detection of *L. infantum*, especially in areas of endemicity (35), it is most likely the more sensitive method for the detection of animal exposure to sandflies bites and/or infection.

Accordingly, the results of the annual incidence of *Leishmania* infection, calculated in March 2006 and March 2007, varied depending on the test employed, with the highest values being registered for PCR (i.e., 5.7% and 11.4% in March 2006 and March 2007, respectively), followed by ELISA, IFAT, and DS. However, by calculating the SE_{rel} and SP_{rel} values for the serological tests on the basis of the results of the PCR with skin tissue, the SE_{rel} values were always very low, while the SP_{rel}

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values were good (from 92 to 100%; Table 4). As a consequence, serological tests are not optimal for the detection of exposed or newly infected animals. Indeed, since dogs infected with *L. infantum* (especially if they are asymptomatic) might not seroconvert immediately after infection or they may develop an immune responsiveness oriented toward a self-healing cellular immune reaction, serological tests may lead to false-negative results.

From a parasitological standpoint, the molecular detection of *L. infantum* in skin samples may indicate prior exposure to infected phlebotomine sandfly bites or an active infection in resistant (i.e., immunocompetent) dogs, or both (38).

This issue remains one of the crucial and most debated aspects of the diagnosis of CanL in asymptomatic dogs. Indeed, a dog which has been bitten by one or more *L. infantum*-infected sandflies should be considered an individual which has been exposed and which needs to be monitored through follow-up. Similarly, in a previous study, 37% of seronegative asymptomatic dogs from an area of endemicity were positive by the PCR with skin tissue, likely as a consequence of effective cellular immunity (37). *L. infantum* promastigotes start to develop in macrophage cells at the site of inoculation in its amastigote form, and the infection may spread, resulting in a systemic form (26). At this stage, an antibody-mediated immune response should occur, but this occurs according to the individual's immune reactivity (3). This infection pattern could explain why at the first follow-up on November 2005, 10 animals were positive only by the *Leishmania* PCR test. Although an animal whose skin tissue samples are positive by PCR must be considered exposed, neither serological nor molecular tests may predict if the animal will remain asymptomatic over the long term, if the animal will clear the infection, or if the animal will exhibit progressive leishmaniasis. Accordingly, three animals which were positive for *Leishmania* only at the first screening converted to a negative result at the second screening, likely because they cleared the infection. A similar picture was previously recorded in asymptomatic animals whose bone marrow was positive by nested PCR and that converted to negativity at the follow-up (27).

Of the 55 animals positive by least at one test performed for *L. infantum* (Table 5), only 7 (12.7%) showed clinical signs related to CanL on March 2007, while the remaining 48 (87.3%) were all asymptomatic. These data fit nicely with the percentage of asymptomatic dogs recorded in Brazil (i.e., 85.3%) (7). Although the clinical appearance of symptoms cannot be ruled out in the follow-up of *L. infantum*-infected animals, the results of the present survey confirm the high prevalence of asymptomatic dogs in areas of endemicity and pinpoint their potential role in the spread of the disease to receptive hosts. Infected asymptomatic animals contribute to the maintenance of the endemicity of the disease by transmitting *Leishmania* spp. to new hosts (dogs or humans) via sandfly bites (22). Under these circumstances, monitoring of asymptomatic infected animals may be of relevance not only for the epidemiological evaluation of CanL in areas of endemicity but also for the control of the infection in human and dog populations by preventing sandfly bites on both infected and uninfected hosts.

The data presented here clearly show that there is no gold standard for the detection of *Leishmania* infections in asymptomatic dogs and highlight the difficulty of proposing a clear diagnostic scheme for assessment of the infection status of asymptomatic dogs. Therefore, more than one test should be used for the diagnosis of CanL in areas of endemicity.

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